Biochem. J. (1980) **188**, 643–648
Printed in Great Britain

Deficient Metabolic Utilization of Hydrogen Peroxide in *Trypanosoma cruzi*

Alberto BOVERIS, Helmut SIES,* Elba E. MARTINO, Roberto DOCAMPO, Julio F. TURRENS and Andrés O. M. STOPPANI†
Instituto de Química Biológica, Facultad de Medicina, Universidad de Buenos Aires, Paraguay 2155, 1121 Buenos Aires, Argentina

(*Received 29 October 1979*)

The glutathione peroxidase–glutathione reductase system, an alternative pathway for metabolic utilization of H_2O_2 [Chance, Sies & Boveris (1979) *Physiol. Rev.* **59**, 527–605], was investigated in *Trypanosoma cruzi*, an organism lacking catalase and deficient in peroxidase [Boveris & Stoppani (1977) *Experientia* **33**, 1306–1308]. The presence of glutathione (4.9 ± 0.7 nmol of reduced glutathione/10^8 cells) and NADPH-dependent glutathione reductase (5.3 ± 0.4 munit/10^8 cells) was demonstrated in the cytosolic fraction of the parasite, but with H_2O_2 as substrate glutathione peroxidase activity could not be demonstrated in the same extracts. With t-butyl hydroperoxide or cumene hydroperoxide as substrate, a very low NADPH-dependent glutathione peroxidase activity was detected (equivalent to 0.3–0.5 munit of peroxidase/10^8 cells, or about 10% of glutathione reductase activity). Blank reactions of the glutathione peroxidase assay (non-enzymic oxidation of glutathione by hydroperoxides and enzymic oxidation of NADPH) hampered accurate measurement of peroxidase activity. The presence of superoxide dismutase and ascorbate peroxidase activity in, as well as the absence of catalase from, epimastigote extracts was confirmed. Ascorbate peroxidase activity was cyanide-sensitive and heat-labile, but no activity could be demonstrated with diaminobenzidine, pyrogallol or guaiacol as electron donor. The summarized results support the view that *T. cruzi* epimastigotes lack an adequate enzyme defence against H_2O_2 and H_2O_2-related free radicals.

The intermediates of the partial reduction of oxygen, $O_2^{-\cdot}$ and H_2O_2, are generated at a multiplicity of subcellular sites by side reactions of physiological electron transfer in the membrane-bound multienzyme redox systems (Chance *et al.*, 1979). Production of $O_2^{-\cdot}$ and H_2O_2 can lead, by a chelated-iron-catalysed reaction, to the generation of the highly oxidizing hydroxyl radical ($HO^{\cdot}$) (Cohen, 1977; McCord & Day, 1978; Halliwell, 1978), which could be the initiator of the free-radical reaction chain of lipid peroxidation. This latter process may result in severe membrane damage, enzyme inactivation and cell death (Chance *et al.*, 1979). The ability of organisms to prevent the lethal action of oxygen-reduction intermediates depends on their content of superoxide dismutase (EC 1.15.1.1), catalase (EC 1.11.1.6) and peroxidases (EC 1.11.1.7). Among the latter enzymes the role of glutathione peroxidase (EC 1.11.1.9) (Mills, 1957) has recently been acknowledged, since in cells such as the rat hepatocyte the enzyme is chiefly responsible for the decomposition of H_2O_2 generated in the cytosol and the mitochondria (Chance *et al.*, 1979).

Mitochondrial, microsomal and cytosolic enzymes from *Trypanosoma cruzi* (the agent of Chagas's disease) generate H_2O_2 at fairly high rates, total H_2O_2 production accounting for at least 4% of the oxygen uptake of respiring *T. cruzi* epimastigotes (Boveris & Stoppani, 1977). *T. cruzi* epimastigotes contain superoxide dismutase (Boveris & Stoppani, 1977), but, like the African trypanosomes (Fulton & Spooner, 1956), they lack catalase (Docampo *et al.*, 1976*b*). This special trait has long been regarded as defining a target for trypanocidal drug design through increased intracellular H_2O_2 steady-state concentrations (Fulton & Spooner, 1956; Kusel *et al.*, 1973; Docampo *et al.*, 1976*b*; Boveris & Stoppani, 1977; Boveris *et al.*, 1978*a*,*b*; Meshnick *et al.*, 1977, 1978; Editorial, 1978). No information is available on glutathione peroxidases in *T. cruzi*, and, therefore, taking into account the importance of H_2O_2 metabolism for the eventual

* Present address: Institut für Physiologische Chemie I, Universität Düsseldorf, Moorenstrasse 5, D-4000 Düsseldorf, German Federal Republic.
† To whom all correspondence should be addressed.

0306-3283/80/060643-06$01.50/1

development of effective chemotherapeutic agents for *T. cruzi*, we have examined the possibility of H_2O_2 utilization through the glutathione peroxidase–glutathione reductase system in the culture form of the parasite.

Materials and Methods

T. cruzi epimastigotes were obtained from liquid cultures (Warren, 1960). Epimastigote homogenates were prepared from epimastigote suspensions (30mg of cell protein/ml) in 50mM-potassium phosphate buffer/1mM-EDTA, pH 7.0. After addition of digitonin (1mg/ml), samples were left at room temperature (approx. 25°C) for 10min, with intermittent shaking in a Vortex mixer. The homogenates were then centrifuged at 8000*g* for 3min in an Eppendorf centrifuge (model 2412), and the supernatant, containing about 9.0mg of protein/ml (30% of total protein in the homogenate), was saved for the experiments. This procedure yielded higher enzyme specific activities than those obtained after other cell-disruption methods, such as freezing and thawing, grinding the cells with glass beads in a mortar and sonication. Glutathione reductase (EC 1.6.4.2) activity was measured spectrophotometrically at 340nm in the presence of 0.1mM-NADPH and 1mM-oxidized glutathione (Nishiki *et al.*, 1976). Total glutathione was measured by the 5,5′-dithio-(2-nitrobenzoic acid) assay method (Tietze, 1969) in 0.25M-$HClO_4$ cell extracts, neutralized with 3M-KOH and 1.5M-triethanolamine. Catalase activity was measured spectrophotometrically at 240nm, by monitoring H_2O_2 (1.0–2.0mM) absorption (Chance, 1954; Docampo *et al.*, 1976*b*). Ascorbate peroxidase activity was determined spectrophotometrically at 265nm in the presence of 50μM-ascorbate and 0.1mM-H_2O_2 (Docampo *et al.*, 1976*b*). Superoxide dismutase activity was measured by determining the inhibition of adrenochrome formation, monitored at 480–575nm, in the presence of 1mM-adrenaline, 0.2mM-xanthine and 1μg of xanthine oxidase/ml (Misra & Fridovich, 1972; Boveris & Stoppani, 1977). NADPH oxidase and glyceraldehyde 3-phosphate dehydrogenase activities were measured spectrophotometrically at 340nm in the presence of 2mM-glyceraldehyde 3-phosphate and either 0.1mM-NADPH or 0.1mM-NAD^+ (Docampo *et al.*, 1976*a*). All enzymic assays were performed in 50mM-potassium phosphate buffer, at 28°C, and measurements were made with a Gilford model 2000 spectrophotometer. Protein was measured by the Folin–Ciocalteu reaction (Lowry *et al.*, 1951), with serum albumin as standard; 1mg of total epimastigote protein corresponded to 0.8×10^8 cells and to 12mg wet wt.

Reagents

Glutathione (oxidized and reduced forms), yeast glutathione reductase, ascorbate (sodium salt), diaminobenzidine, guaiacol, pyrogallol, digitonin, glyceraldehyde 3-phosphate, NAD^+ and NADPH (Sigma Chemical Co., St. Louis, MO, U.S.A.), cumene hydroperoxide (Matheson, Coleman and Bell, Norwood, OH, U.S.A.) and t-butyl hydroperoxide (Aldrich Co., Milwaukee, WI, U.S.A.) were purchased from the sources indicated. Other reagents were of analytical grade. Erythrocyte glutathione peroxidase was a gift from Dr. A. Wendel, University of Tübingen, Tübingen, Germany.

Results

Determination of glutathione, glutathione reductase activity and glutathione peroxidase activity

Measurement of total glutathione content in nine different epimastigote extracts yielded a value (mean ± S.E.M.) of 4.9 ± 7nmol of GSH/10^8 epimastigotes; this value is about one-tenth of the glutathione content in rat liver cells (Burk *et al.*, 1978) when contents are expressed per g of wet tissue. No attempt was made to differentiate between oxidized and reduced glutathione. The presence of NADPH-dependent glutathione reductase in the cell-free extracts could be demonstrated, as shown in Table 1. The enzyme content in epimastigotes was about one-thirtieth of that in rat liver cells (Nishiki *et al.*, 1976), calculated per g of wet tissue.

Fig. 1 shows an attempt to demonstrate glutathione peroxidase in epimastigote extracts with t-butyl hydroperoxide as substrate. The rate of the reaction was measured by NADPH oxidation. In the first experiment (trace *a*) the extract was added after glutathione reductase, NADPH and t-butyl hydroperoxide. NADPH was slowly oxidized, this reaction being attributed to an NADPH oxidase in the epimastigote extract, since the oxidation did not occur under N_2. Addition of reduced glutathione further increased the rate of the overall reaction (the abrupt downward deflexion that follows addition of reduced glutathione is interpreted as being due to the presence of oxidized glutathione in the sample of reduced glutathione). In the second experiment (trace *b*), addition of extract after NADPH resulted in a slow oxidation of reduced nicotinamide nucleotide (again, the NADPH oxidase reaction); addition of reduced glutathione and glutathione reductase did not modify the rate of NADPH oxidation, but the subsequent addition of t-butyl hydroperoxide increased it to a significant extent. In the third experiment (trace *c*), activity was obtained in the absence of extract, this being interpreted as being due to non-enzymic oxidation of reduced glutathione by t-butyl hydroperoxide; addition of extract

Table 1. *Glutathione reductase, glutathione peroxidase and other enzyme activities in T. cruzi epimastigotes*
Experimental conditions were as described in the legend to Fig. 1 and in the text. The values represent mean values ± S.E.M., with the numbers of individual extracts assayed in parentheses. Activity is expressed in munits of the enzyme corresponding to the specified reaction (1 unit = 1 μmol/min). The presented values include the corrections for the blank reactions (see Fig. 1).

Enzyme reaction	Oxidant	Activity (munits/10^8 cells)
Glutathione reductase	Oxidized glutathione	5.3 ±0.4 (8)
Glutathione peroxidase	H_2O_2	0 ±1.7 (8)
	t-Butyl hydroperoxide	0.39 ±0.09 (22)
	Cumene hydroperoxide	0.55 ±0.28 (6)
NADPH oxidase	O_2	3.8 ±0.2 (8)
Glyceraldehyde 3-phosphate dehydrogenase	NAD^+	56 ±4 (8)

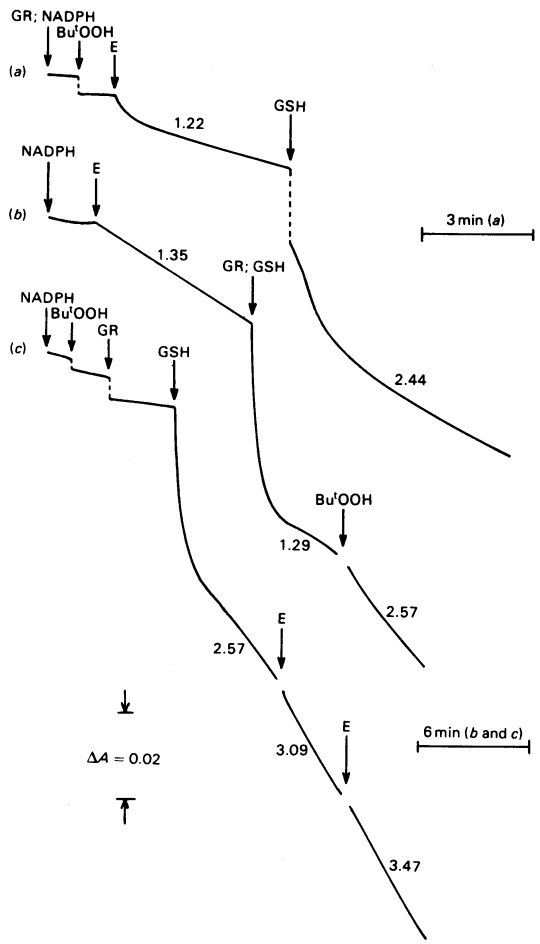

Fig. 1. *Assay of glutathione peroxidase activity in T. cruzi extracts with t-butyl hydroperoxide as oxidant*
The reaction mixture (3.0 ml) contained 50 mM-potassium phosphate buffer, pH 7.0, and the indicated additions: NADPH (0.1 mM); Bu'OOH, t-butyl hydroperoxide (0.1 mM); GR, glutathione reductase (0.2 unit); GSH, reduced glutathione (1.0 mM); E, epimastigote extract (0.16 mg of protein). Other conditions were as described in the text. The values near the tracings indicate the rate of NADPH oxidation, in nmol of NADPH/min.

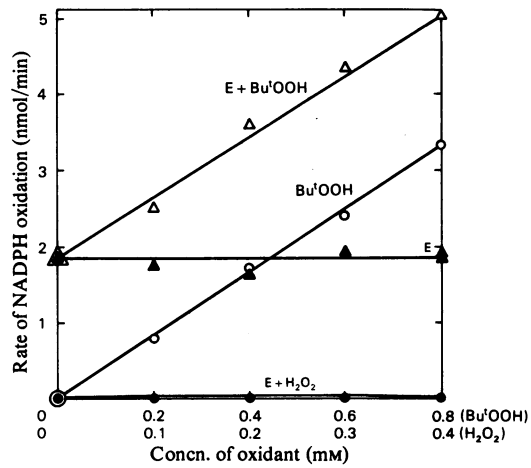

Fig. 2. *Effect of oxidant concentration on glutathione peroxidase activity*
Experimental conditions were as in Fig. 1 (complete system) except for the epimastigote extract (E, 0.2 mg) and oxidant, which concentration is indicated on the abscissa. Bu'OOH, t-Butyl hydroperoxide. Where not indicated, oxidant and extract were omitted.

increased somewhat the rate of NADPH oxidation, in proportion to the extract concentration. It is remarkable that, in all the selected experimental conditions, the final rate of NADPH oxidation was relatively small and scarcely exceeded the rate of the blank reaction mixture from which the epimastigote extract was omitted.

Fig. 2 shows glutathione peroxidase activity as a function of the concentrations of added oxidant (t-butyl hydroperoxide and H_2O_2). With H_2O_2 as substrate no glutathione peroxidase could be detected, but with t-butyl hydroperoxide activity increased linearly as a function of oxidant concentration. It is worth noting, however, that (*a*) a parallel increase was observed with the non-enzymic oxidation of glutathione by t-butyl hydroperoxide

(the second-order rate constant of this reaction was $7 \times 10^{-3} M^{-1} \cdot s^{-1}$) and (*b*) the increase of apparent peroxidase activity induced by the epimastigote extract was accounted for by the NADPH oxidase activity of this extract. Similar experiments were performed with several epimastigote extracts and also with cumene hydroperoxide as substrate. The mean ± S.E.M. values are presented in Table 1. With all the assayed hydrogen acceptors the S.E.M. was relatively large, which is not surprising considering that these values involve the propagation of the blank-reactions error. With H_2O_2 as substrate no glutathione peroxidase could be demonstrated. With t-butyl hydroperoxide and cumene hydroperoxide the average value of the apparent peroxidase activity was very low, about 1/2500 the activity in rat hepatocytes (Burk *et al.*, 1978). Moreover, the ratio of the t-butyl hydroperoxide-elicited activity to the glutathione reductase activity in extracts was less than 0.1, as opposed to 1.95 for the same ratio in the rat hepatocyte (Sies *et al.*, 1979).

Investigation of glutathione peroxidase activity was repeated with extracts obtained from epimastigotes treated with 2.0 or 0.5 mg of digitonin/ml, treated with 1% (v/v) Triton X-100, or subjected to freezing–thawing before extraction with the phosphate/EDTA buffer (see the Materials and Methods section). Furthermore, to rule out the possibility of enzyme inactivation by digitonin treatment during epimastigote homogenization, cytosolic H_2O_2-generating NADPH oxidase and glyceraldehyde 3-phosphate dehydrogenase activities were measured in the epimastigote extracts. These enzyme activities (Table 1) were in accordance with results obtained by Boveris & Stoppani (1977) and Docampo *et al.* (1976*a*) respectively, with extracts prepared by methods excluding digitonin. The possibility of a glutathione peroxidase inhibitor in epimastigotes was investigated by measuring the activity of pure erythrocyte glutathione peroxidase (143 nmol of NADPH oxidized/min) dissolved in the epimastigote extract. The measured activity was 96% of the control one, thus ruling out the presence of a peroxidase inhibitor. Finally, the reliability of the glutathione peroxidase assay system was also tested by using rat liver extract as source of glutathione peroxidase. After this latter addition, NADPH was rapidly oxidized at a rate more than twice the rate with the epimastigote extract (results not shown).

Assay of other H_2O_2-metabolizing enzymes

To complete the picture of peroxide-metabolizing enzymes in *T. cruzi*, superoxide dismutase, catalase and ascorbate peroxidase were investigated in the extracts used for the glutathione peroxidase assay. Values for these enzyme activities, as in Table 2, confirm reports by Docampo *et al.* (1976*b*) and Boveris & Stoppani (1977). With regard to superoxide dismutase, the content was about one-twentieth of the enzyme content in rat hepatocytes (Tyler, 1975). Because of the apparent importance of ascorbate peroxidase for the utilization of H_2O_2 in *T. cruzi*, this activity was assayed with electron donors other than ascorbate (diaminobenzidine, pyrogallol, guaiacol and reduced cytochrome *c*) and also in the presence of cyanide. The results in Table 2 show that no peroxidase activity could be detected except with ascorbate. Cyanide inhibited ascorbate peroxidase activity, but at a relatively high concentration as compared with typical peroxidases. Ascorbate peroxidase activity was heat-labile, since complete inactivation was obtained after 5 min at 100°C. Moreover, activity was lost after dialysis of epimastigote extracts (results not shown).

Table 2. *H_2O_2-metabolizing enzymes in T. cruzi epimastigotes*

Experimental and other conditions were as described in the text and in Table 1. Enzyme activities are expressed as in Table 1, except superoxide dismutase. One superoxide dismutase unit is the amount of enzyme that inhibits by 50% the reduction of 10 μM-cytochrome c^{3+} by 0.1 mg of xanthine oxidase/ml in the presence of 0.2 mM-xanthine (McCord & Fridovich, 1969).

Enzyme reaction	Substrate (μM) and inhibitor (mM)	Activity (munits/10^8 cells)
Peroxidase	H_2O_2 (100); ascorbate (50)	7.5 ± 1.2 (4)
	Same + KCN (1)	5.0 (2)
	Same + KCN (3)	3.0 (2)
	Same + KCN (7)	1.0 (2)
	H_2O_2 (100); diaminobenzidine (50)	0 (4)
	H_2O_2 (100); guaiacol (30)	0 (4)
	H_2O_2 (100); pyrogallol (50)	0 (4)
	H_2O_2 (100); cytochrome c^{2+} (50)	0 (4)
Catalase	H_2O_2 (1000)	0 (6)
Superoxide dismutase	$O_2^{-\cdot}$	68 ± 7 (4)

Discussion

The present observations confirm that *T. cruzi* is an organism poorly endowed to detoxify H_2O_2, since, in addition to the absence of catalase (Table 2), no H_2O_2-utilizing glutathione peroxidase activity could be demonstrated in epimastigote extracts (Fig. 2). With regard to the very low glutathione peroxidase activities measured with the organic hydroperoxides, these may reflect a relatively non-specific reaction, such as the one catalysed by glutathione *S*-transferase B (Burk *et al.*, 1978), where oxidation of reduced glutathione by hydroperoxides occurs at hydrophobic areas of the protein. Accordingly, ascorbate peroxidase (Docampo *et al.*, 1976*b*; Table 2) should be the sole biochemical mechanism capable of metabolizing H_2O_2 in *T. cruzi*. Nevertheless, the inactivity observed with substrates used by typical peroxidases, such as diaminobenzidine, pyrogallol and guaiacol, casts some doubt on the physiological significance of ascorbate peroxidase activity, and leads one to consider the possibility that ascorbate oxidation may be due to a protoenzyme-like complex of metal loosely bound to a protein present in *T. cruzi* epimastigotes. Inhibition of ascorbate peroxidase activity by cyanide (Table 2), as well as the heat-lability of the involved factor, lend experimental support to that hypothesis.

McCord *et al.* (1971) have noted that aerobic bacteria contain both superoxide dismutase and catalase, and that aerotolerant anaerobes have ample superoxide dismutase and low catalase activities, whereas obligatory anaerobes appear to lack both enzymes. In this context, *T. cruzi* epimastigotes could be regarded as aerotolerant anaerobes, but, at variance with that assumption, inhibition of mitochondrial electron transfer by antimycin A is lethal for *T. cruzi* epimastigotes (Boiso *et al.*, 1979), thus indicating the organisms' dependence on aerobic energy.

The apparent absence of catalase and glutathione peroxidase (H_2O_2-dependent), as well as the very low activity and uncertain function of ascorbate peroxidase, make *T. cruzi* epimastigotes particularly liable to the toxic action of H_2O_2. These enzyme deficiencies suffice to explain the trypanocidal effects on *T. cruzi* of (*a*) β-lapachone (Docampo *et al.*, 1978; Boveris *et al.*, 1978*a*,*b*), (*b*) the peroxidase I/glucose oxidase mixtures (Avila *et al.*, 1978) and (*c*) activated macrophages (Nathan *et al.*, 1979). The same cytotoxic mechanism may hold for the African trypanosomes (e.g. *T. brucei*), where haem and related porphyrins have proved to be trypanocidal, probably through $HO^{\bullet}$ generation from H_2O_2, and where synergism between porphyrins and H_2O_2-generating naphthoquinones has been reported (Meshnick *et al.*, 1977, 1978). The possibility of turning the abnormal H_2O_2 metabolism of parasite trypanosomes to therapeutic advantage constitutes a novel and important biochemical alternative (Editorial, 1978). Nifurtimox, one of very few available agents for the treatment of Chagas's disease, induces $O_2^{-\bullet}$ and H_2O_2 production in *T. cruzi* epimastigotes, at extracellular concentrations compatible with those in blood after clinical administration of the drug (Docampo & Stoppani, 1979).

This work was supported by grants from the Secretary of State of Science and Technology (Program of Chagas's Disease), República Argentina, the Scientific Office of the Organization of American States and the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases. Collaboration of H. S. was facilitated by the Deutscher Akademische Austauschdienst (German Federal Republic) and National Research Council (CONICET), República Argentina. A. B., R. D. and A. O. M. S. are Career Investigators and E. E. M. and J. F. T. are Research Fellows of CONICET.

References

Avila, J. L., Bretaña, A., Jacques, P. J., Casanova, M. A. & Convit, J. (1978) in *Current Chemotherapy, Proc. Int. Congr. Chemother. 10th* (Siegenthaler, W. & Lüthy, R., eds.), pp. 153–155, American Society for Microbiology, Washington

Boiso, J. F., Docampo, R., Stoppani, A. O. M. & Frasch, A. C. C. (1979) *Medicina (Buenos Aires)* **39**, 362–368

Boveris, A. & Stoppani, A. O. M. (1977) *Experientia* **33**, 1306–1308

Boveris, A., Docampo, R., Turrens, J. F. & Stoppani, A. O. M. (1978*a*) *Biochem. J.* **175**, 431–439

Boveris, A., Stoppani, A. O. M., Docampo, R. & Cruz, F. S. (1978*b*) *Comp. Biochem. Physiol. B* **61**, 237–239

Burk, R. F., Jr., Nishiki, K., Lawrence, R. A. & Chance, B. (1978) *J. Biol. Chem.* **253**, 43–46

Chance, B. (1954) *Methods Biochem. Anal.* **1**, 412–424

Chance, B., Sies, H. & Boveris, A. (1979) *Physiol. Rev.* **59**, 527–605

Cohen, G. (1977) in *Superoxide and Superoxide Dismutases* (Michelson, A. M., McCord, J. M. & Fridovich, I., eds.), pp. 317–321, Academic Press, London and New York

Docampo, R. & Stoppani, A. O. M. (1979) *Arch. Biochem. Biophys.* **197**, 317–321

Docampo, R., Boiso, J. F. & Stoppani, A. O. M. (1976*a*) *Rev. Asoc. Argent. Microbiol.* **8**, 21–25

Docampo, R., Boiso, J. F., Boveris, A. & Stoppani, A. O. M. (1976*b*) *Experientia* **32**, 972–975

Docampo, R., Cruz, F. S., Boveris, A. Muñiz, R. P. A. & Esquivel, D. M. S. (1978) *Arch. Biochem. Biophys.* **186**, 292–297

Editorial (1978) *Lancet* **ii**, 1190

Fulton, J. D. & Spooner, D. F. (1956) *Biochem. J.* **63**, 475–481

Halliwell, B. (1978) *FEBS Lett.* **92**, 321–326

Kusel, J. P., Boveris, A. & Storey, B. T. (1973) *Arch. Biochem. Biophys.* **158**, 799–805

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
McCord, J. M. & Day, E. M., Jr. (1978) *FEBS Lett.* **86**, 139–142
McCord, J. M. & Fridovich, I. (1969) *J. Biol. Chem.* **244**, 6049–6055
McCord, J. M., Keele, B. & Fridovich, I. (1971) *Proc. Natl. Acad. Sci. U.S.A.* **68**, 1024–1027
Meshnick, S. R., Chang, K.-P. & Cerami, A. (1977) *Biochem. Pharmacol.* **26**, 1923–1928
Meshnick, S. R., Blobstein, S. H., Grady, R. W. & Cerami, A. (1978) *J. Exp. Med.* **148**, 569–579
Mills, G. C. (1957) *J. Biol. Chem.* **229**, 189–197
Misra, H. P. & Fridovich, I. (1972) *J. Biol. Chem.* **247**, 3170–3175
Nathan, C., Nogueira, N., Juangbhanich, C., Ellis, J. & Cohn, Z. (1979) *J. Exp. Med.* **149**, 1056–1068
Nishiki, K., Oshino, N., Jamieson, D. & Chance, B. (1976) *Biochem. J.* **160**, 343–355
Sies, H., Koch, O. R., Martino, E. & Boveris, A. (1979) *FEBS Lett.* **103**, 287–290
Tietze, F. (1969) *Anal. Biochem.* **27**, 502–522
Tyler, D. D. (1975) *Biochem. J.* **147**, 493–504
Warren, L. (1960) *J. Parasitol.* **46**, 529–539